Physiological, Genetic and Proteomic Characterization of *Arthrobacter chlorophenolicus* During Growth on Different Phenolic Substrates or Temperatures

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Abstract

The species *Arthrobacter chlorophenolicus* was isolated from soil because of its ability to degrade high concentrations of 4-chlorophenol and other toxic aromatic compounds. It is also able to grow on and degrade phenolic compounds at low temperatures as well as during extreme temperature fluctuations. In this thesis the degradation pathway through which 4-chlorophenol is degraded by this organism is described. It is an efficient and very unusual 4-chlorophenol catabolic pathway for aerobic bacteria, with hydroxyquinol as the ring-cleavage substrate.

In addition, *A. chlorophenolicus* was studied during growth on mixtures of phenolic compounds, giving new insights into substrate preferences and mechanisms behind the sequential degradation of similar compounds. Changes in cell membrane fatty acid composition at two different temperatures when phenolic compounds were added in increasing concentrations were also studied. The results have provided more information about this adaptive response in Gram positive bacteria. In addition, the proteins expressed during the same growth conditions mentioned above were identified by shotgun proteomics, confirming the catabolic routes used by this organism during growth on phenolic compounds as well as some of its stress response mechanisms. The genome sequence of *A. chlorophenolicus* was sequenced and annotated, revealing secrets of adaptation and stress mechanisms as well as new findings on the genome structure and catabolic activities of *A. chlorophenolicus*.

Keywords: *Arthrobacter chlorophenolicus*, 4-chlorophenol, 4-nitrophenol, phenol, hydroxyquinol, bioremediation, shotgun proteomics

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Fysiologi, gener och proteiner hos *Arthrobacter chlorophenolicus* under nedbrytning av olika fenoler och vid olika temperaturer.

Sammanfattning

Bakterien *Arthrobacter chlorophenolicus* isolerades från jord på grund av sin förmåga att bryta ner höga halter 4-klorfenol och andra giftiga aromatiska (ring-formade) ämnen. Dess nedbrytning av flera olika fenoler kan även ske under svåra förhållanden, såsom i låga temperaturer eller under extrema temperaturväxlingar.

Nedbrytningskedjan som *A. chlorophenolicus* använder för att växa på 4-klorfenol är effektiv och ovanlig hos andra aeroba (syrekrävande) bakterier. Den centrala metaboliten i nedbrytningsvägen är hydroxyquinol, det är den aromatiska molekyl som bryts sönder till en rak kedja. *A. chlorophenolicus* har bland annat studerats under tillväxt på en blandning av olika fenoler. Denna studie gav ny kunskap om hur ämnena påverkade varandras nedbrytning och bakterien i sig, i vilken ordning ämnena bröts ner och vilka mekanismer som kan ligga bakom en sådan nedbrytningsordning. En annan studie behandlade cellmembranets anpassningar till ökande koncentrationer av olika fenoler och till olika temperaturer, vilket ledde till ny kunskap om denna process hos Gram-positiva bakterier. Även de proteiner som uttrycktes vid tillväxt på de olika fenolerna och vid de olika temperaturerna studerades med hjälp av en s.k. shotgun proteomics-metod. De många skillnader som fanns mellan proteinuttryck under de olika förhållandena bekräftade flera tidigare kända anpassningar till svåra miljöer, och visade även på proteiner som inte tidigare anknutits till tillväxt i kyla eller i närvaro av olika fenoler. Tidigare teorier om nedbrytningsvägar i *A. chlorophenolicus* kunde till viss del också bekräftas.

Även *A. chlorophenolicus* alla gener sekvenserades, och nya rön om genomets struktur, anpassningsmekanismer och möjligheter att bryta ner olika ämnen kunde utläsas.

Tillägnas mormor

var du än är

Att somliga har vingar
och sitter i träden
och sjunger –
visst är det en ganska fantastisk idé?

- Ingrid Sjöstrand -

Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Nordin, K., M. Unell, & J. K. Jansson. (2005). Novel 4-chlorophenol degradation gene cluster and degradation route via hydroxyquinol in *Arthrobacter chlorophenolicus* A6. *Applied and Environmental Microbiology* 71, 6538-6544.

II Unell, M., K. Nordin, C. Jernberg, J. Stenström & J. K. Jansson (2007). Degradation of phenol mixtures by *Arthrobacter chlorophenolicus*. *Biodegradation*. In press.

III Unell, M., N. Kabelitz, J. K. Jansson & H. J. Heipieper (2007). Adaptation of the psychrotroph *Arthrobacter chlorophenolicus* A6 to growth temperature and the presence of phenols by changes in the *anteiso/iso* ratio of branched fatty acids. *FEMS Microbiology Letters* 266, 138-143.

IV Unell, M., P. E. Abraham, M. Shaw, B. Zhang, N. VerBerkmoes & J. K. Jansson. Proteomic analysis of *Arthrobacter chlorophenolicus* grown on different phenol substrates and growth temperatures (manuscript).

Papers I-III are reproduced with the permission of the publishers.

Abbreviations

4-CP	4-chlorophenol
4-NP	4-nitrophenol
4-BP	4-bromophenol
PAH	Polycyclic aromatic hydrocarbon
DETP	Diethylthiophosphoric acid
FAME	Fatty Acid Methyl Ester
2D-PAGE	2-dimensional Polyacrylamide Gel Electrophoresis
ESI	Electrospray Ionization
MALDI	Matrix Assisted Laser Desorption/Ionization

Tänk på allt du aldrig ser –
Trädets växande.
Och fisken som vandrar.
Grodans sömn i kärret
under vinterfrosten.
En liten prick på himlen
är en örn.
Och mossan kryper under gärdet.
I en stad
sitter en man med en skruvmejsel.

Tänk på allt du aldrig ser –
Det finns där.
Alltsammans.

- Einar Økland -

Introduction

A teaspoon of soil can easily contain billions of bacteria. In our bodies there are more bacterial cells than human cells - they constitute approximately 2 kg of our weight. The microbial world is a silent, invisible universe to our naked eyes and ears, yet it has a tremendous impact on life on earth.

In the work of this thesis I have come to know more about one of these bacterial species, *Arthrobacter chlorophenolicus*. It is a soil bacterium belonging to the genus *Arthrobacter*, one of the most abundant culturable bacteria found in soil. Members of this genus are known for high resistance to desiccation, starvation and other stresses, although they do not form spores. Spores are otherwise an efficient way for bacteria to survive harsh conditions. The *Arthrobacter* genus is also a heterogeneous group that can degrade many different compounds (Madigan, 2000).

Members of the genus *Arthrobacter* have various strategies to survive stressful conditions, which is a necessity if you are not comfortably laid back in a laboratory Petri dish. Their living room in nature is the soil we walk on, and this is not a very relaxing environment. Large fluctuations in moisture and temperature occur in most temperate soils. In addition, some soils are polluted by potentially toxic substances such as pesticides, petroleum, heavy metals and solvents. These substances can also cause immense stress to microscopic soil inhabitants. There are also other microorganisms competing for life "down-under", secreting antibiotics and digesting available energy resources. Starvation is often just around the corner, and protozoa lurk under every grain to prey on smaller bacterial cells. *Arthrobacter* species have evolved several mechanisms to survive many of these sources of stress. For example, many species can produce substances such as trehalose and glycogen to protect them from osmotic stress (Zevenhuizen, 1992, Mongodin *et al.*, 2006). In addition, *Arthrobacter* species switch between two physiological states during their lifetime: the rod-shaped cell, associated with

active growth, and the coccoid cell during stationary phase (Fig. 1). The latter, spherically shaped cell is more resistant to stress, and it is in this form that *Arthrobacter* species normally survive long periods of drought, cold or starvation.

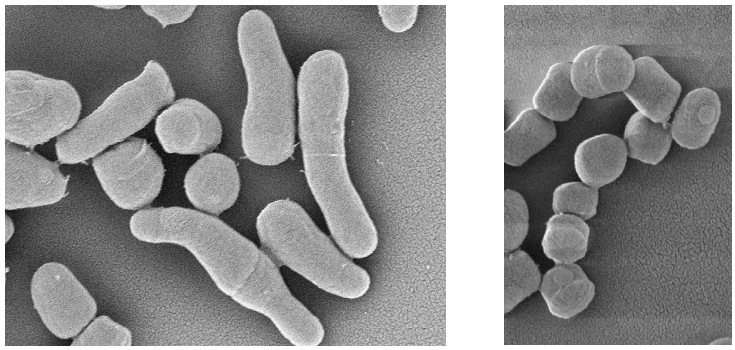

Figure 1. Rod-shaped and coccoid cells of *Arthrobacter chlorophenolicus*.

Cold and other forms of stress induce the formation of cold-shock proteins and other stress-related proteins with different roles in the complex machinery of the cell.

In order to compete with other organisms for the same niche in soil, antibiotic biosynthesis as well as antibiotic resistance can evolve. To survive the presence of harmful substances such as pollutants, solvents and heavy metals some bacteria have evolved the ability to degrade or modify the toxic compounds, and many microorganisms can also actively export harmful substances out of the cell. In addition, motile bacteria such as *A. chlorophenolicus* have the ability to simply move away from unpleasant environments if permitted by humidity, pore size etc.

Many of the toxic substances in soil and water have been created and released in nature by humans. The extensive use of pesticides in the last century has resulted in vast pollution of soil and water. These man-made substances are called xenobiotic compounds. However, thanks to the impressive adaptation capacity of microorganisms, these toxic compounds can often serve as carbon, nitrogen and/or energy sources for many of them. The *Arthrobacter* genus is recognized for its nutritional versatility, and some members of the genus can degrade for example nicotine (Schenk *et al.*,

1998), the herbicide 2,4-dichlorophenoxyacetate (Tiedje *et al.*, 1969), glyphosate (Pipke *et al.*, 1987), polycyclic aromatic hydrocarbons (PAHs) such as phenanthrene (Kallimanis *et al.*, 2007), pentachlorophenol (Lin & Wang, 1991), halogenated phenols such as 4-nitrophenol (Perry & Zylstra, 2007, **Paper I** and **II**) and several s-triazine herbicides (Strong *et al.*, 2002).

The ability to use normally toxic substances for growth does not rule out the fact that the compounds are still potentially inhibitory to cell growth. It takes energy for a bacterium to minimize the negative effects that an otherwise toxic compound has on the cell, at the same time it is using it as an energy source. There are several mechanisms by which bacteria adapt to toxic organic compounds. For example, many organic compounds are lipophilic and act as solvents on the cell membrane, requiring the bacterium to adapt its membrane fatty acid composition in order to maintain a functional cell membrane (**Paper III**).

As mentioned in the beginning of this section, the lead actor of this thesis is the soil bacterium *A. chlorophenolicus*. It can grow on and degrade high concentrations of different phenolic compounds, even at temperatures as low as 5°C (Backman & Jansson, 2004). In the scope of this thesis its genetics, degradation pathway(s), as well as its physiological behaviour during degradation of different aromatics, sole and in mixtures, have been described (**Papers I** and **II**). Adaptation mechanisms in the form of physiological changes in the cell membrane and changes in protein expression to high concentrations of phenolic compounds and to low temperatures were specifically studied (**Papers III** and **IV**).

During the progress of this thesis' work the complete genome of *A. chlorophenolicus* was sequenced, and as a consequence it was also possible to explore the complete proteomes of the bacterium during different growth conditions (**Paper IV**). This leap forward in data acquisition has given new insights into stress response mechanisms and catabolic routes used by *A. chlorophenolicus*.

The findings of this thesis have contributed to the knowledge related to biodegradation and stress adaptations by soil bacteria in general, and Gram positive bacteria in particular. They have also given further insights into genomics of the *Arthrobacter* genus and the first description of the expressed protein products of one of its members.

"*…l'essentiel est invisible par les yeux*"

- "Le petit prince", Antoine St Exupéry -

Actinobacteria

The smell of healthy soil, or the smell after a summer rain – this is the smell of actinobacteria. The smell is caused by the secondary metabolite geosmin which in soil is produced primarily by actinobacteria (Gerber & Lecheval, 1965). These organisms are Gram positive bacteria with a high ratio of guanine + cytosine (G+C) nucleotides in their DNA. This phylum is one of the largest taxonomic groups among bacteria in terms of number and variety of identified species. Some of the most common genera found in soil belong to actinobacteria, for example *Actinomyces*, *Streptomyces*, *Rhodococcus*, *Micrococcus* and *Arthrobacter*.

The majority of actinobacteria require oxygen to grow, but they range from obligate aerobic to anaerobic organisms. Actinobacteria are generally very tolerant to drought and starvation, which may be the reason for their abundance in soil. This survival capacity may, at least in some species, partly be explained by the synthesis of unique cell wall peptidoglycans (Gokhale *et al.*, 2007). The phylum is also well known for production of secondary metabolites and antibiotic substances, and members of the genus *Streptomyces* belong to the most prolific antibiotic producers. There are also reports of actinobacteria as endophytic organisms, helping the host plant to suppress pathogens (Conn *et al.*, 2008) or enhance plant-associated remediation of contaminated soil, "phytoremediation" (Moore *et al.*, 2006).

Actinobacteria are important for decomposition of organic matter and formation of humus, and many members of this group are efficient degraders of an array of different organic compounds, including toxic pollutants. This ability can be explained in part by their capacity to transform complex organic substances such as lignin-related compounds occurring in nature (Ball *et al.*, 1989), and their ability to produce biosurfactants or bioemulsifiers (Bicca *et al.*, 1999). In addition, the hydrophobicity of their cell surfaces

allows close contact with, or adhesion to, low water soluble or lipophilic molecules, which favours the uptake process (Bastiaens *et al.*, 2000).

The high survival capacity and diverse degradation abilities of actinobacteria make them very interesting candidates for bioremediation processes in contaminated soil. There are examples of actinobacterial degradation of e.g. PAH (Pizzul *et al.*, 2007), PCB (Seto *et al.*, 1995, Warren *et al.*, 2004) and also chromium (VI) resistance and removal (Polti *et al.*, 2007) in soil.

Arthrobacter

The genus *Arthrobacter* is one of the most common genera found in soil, and almost all species in this genus are obligate aerobes with the shape of rods during exponential growth and cocci in stationary phase. It is distinctive in its use of a "snapping division", in which the outer bacterial cell wall breaks at a joint, causing a snap in the cell division at the time of rupture. The resulting two daughter cells often remain in contact for a long period after division, in a distinct V-shape.

Arthrobacter species have been isolated from almost all soil types. In addition, they have been isolated from harsh environments such as alpine permafrost (Bai *et al.*, 2006) and Antarctic seawater, where they degraded PCB (Luigi *et al.*, 2007). Some species from this genus have even been cultured below the freezing point of water, i.e. down to -17°C (Panikov & Sizova, 2007).

Arthrobacter chlorophenolicus – background

The species *Arthrobacter chlorophenolicus* was isolated from a soil slurry because of its ability to degrade 4-chlorophenol (4-CP) and other toxic aromatic compounds. *A. chlorophenolicus* was adapted to degrade high concentrations (up to 350 ppm in pure culture) of 4-CP as a sole carbon source by selective enrichment (Westerberg *et al.*, 2000, Nordin, 2004), see Fig. 2.

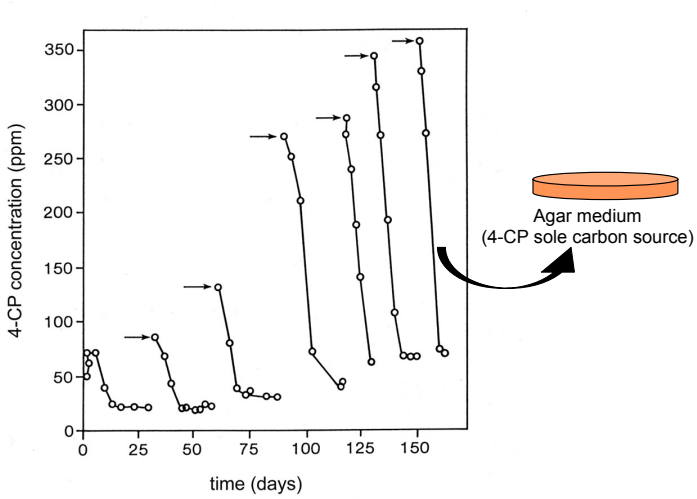

Figure 2. Selective enrichment with increasing concentrations of 4-chlorophenol. Arrows show the time points when the culture was spiked with 4-chlorophenol. Adapted from Westerberg *et al.*, 2001.

In addition to 4-CP, *A. chlorophenolicus* can also degrade other para-substituted mono-aromatics, such as 4-nitro-, 4-bromo- and 4-fluorophenol as well as phenol (Fig. 3). Recent experiments also indicate that *A. chlorophenolicus* can degrade parathion, a very toxic organophosphate compound (Fig. 3).

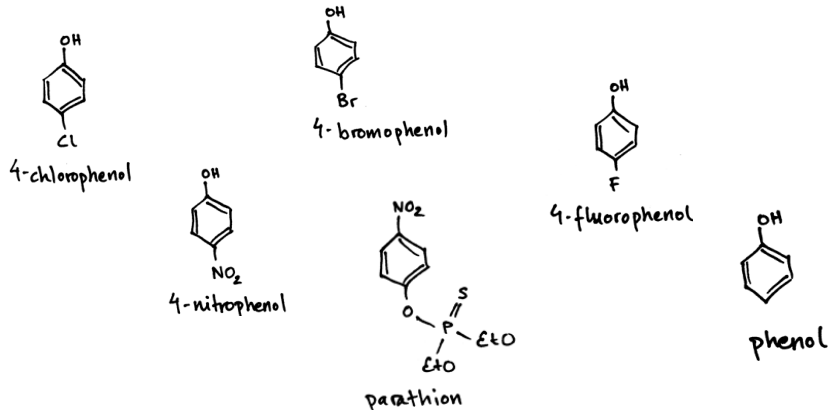

Figure 3. Aromatic compounds degraded by *Arthrobacter chlorophenolicus*.

The 4-CP degradation pathway employed by this species, using hydroxyquinol as the ring cleavage substrate (**Paper I**), has not previously been reported for aerobic bacteria. In **Paper II** we propose that the *A. chlorophenolicus* degradation pathways of substituted phenols converge in hydroxyquinol as the ring cleavage substrate. Later publications of other *Arthrobacter* species degrading 4-nitrophenol (4-NP) and 4-fluorophenol through hydroxyquinol support this theory (Perry & Zylstra, 2007, Ferreira *et al.*, 2008).

A. chlorophenolicus was previously used to bioaugment non-sterile soil microcosms contaminated with high concentrations of 4-CP. The cells were capable of degradation of this compound and therefore showing promise as an agent for soil remediation (Elväng *et al.*, 2001, Jernberg & Jansson, 2002), see Fig. 4. *A. chlorophenolicus* cells are also able to survive and to degrade 4-CP at low temperatures, characteristic for temperate climates, and during extreme fluctuations in temperatures (Backman & Jansson, 2004, Backman *et al.*, 2004).

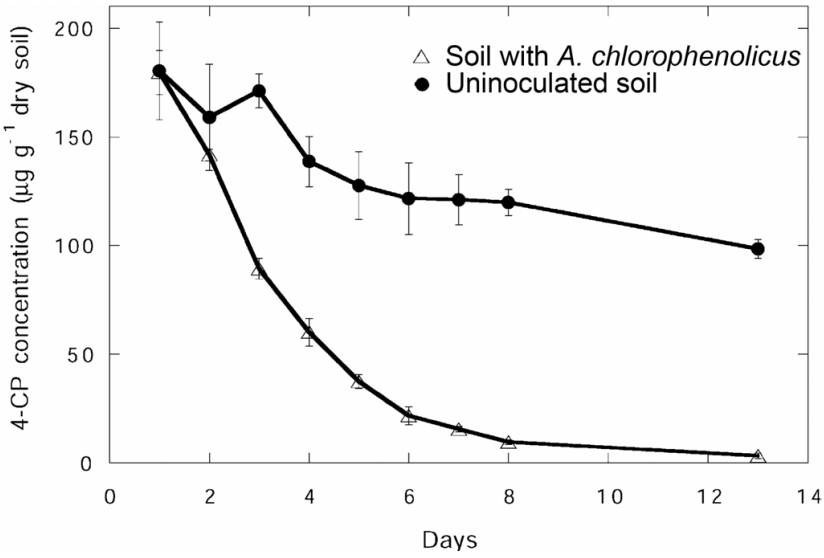

Figure 4. Degradation of 4-CP in soil microcosms (Jernberg & Jansson, 2002), reproduced with permission of the publishers.

Previously, *A. chlorophenolicus* was tagged with marker genes, namely *gfp*, encoding the green fluorescent protein (GFP), resulting in strain *A. chlorophenolicus* A6G, and *luc*, encoding firefly luciferase, resulting in strain *A. chlorophenolicus* A6L. These tagged strains have been used to specifically monitor *A. chlorophenolicus* cells in non-sterile soil (Elväng *et al.*, 2001). In addition, the A6L strain was used to study the impact of temperature on the physiological status of *A. chlorophenolicus* cells, showing better survival in soil at lower temperatures (5°C), compared to 28°C (Backman *et al.*, 2004). The A6L strain was also used to monitor metabolic activity of the cells during degradation of mixtures of phenolic compounds (**Paper II**).

A. chlorophenolicus is also capable of effectively degrading and growing on some phenolic compounds as its sole source of carbon and energy, and in the case of 4-NP also as its nitrogen source, even at low temperatures. This mission is completed solely by *A. chlorophenolicus* on its own, not through co-metabolism and without requiring any additional nutrients or inducing compounds to start the degradation process. In addition, the high survival of *A. chlorophenolicus* cells at low temperatures, and its tolerance to temperature fluctuations, starvation, drought and other stresses makes this microorganism very interesting to study with respect to survival mechanisms in general. These were the basic fundaments on which this thesis' work was based.

We have forgotten how to be good guests – how to walk lightly on earth as its other creatures do.

– Stockholm conference, Only One Earth –

Aromatic Pollutants

The aromatic ring of cyclic contaminants is not in itself foreign to nature, for example it is found in lignin (Fig. 5). However, in the hands of humans numerous compounds can be substituted, or added to the ring(s), creating substances that are foreign to nature and that may have increased toxicity. These man-made compounds are called xenobiotic, or foreign to life. Examples of such compounds are polycyclic aromatic hydrocarbons (PAH), dioxin, polychlorinated biphenyls (PCB) and many chlorophenols.

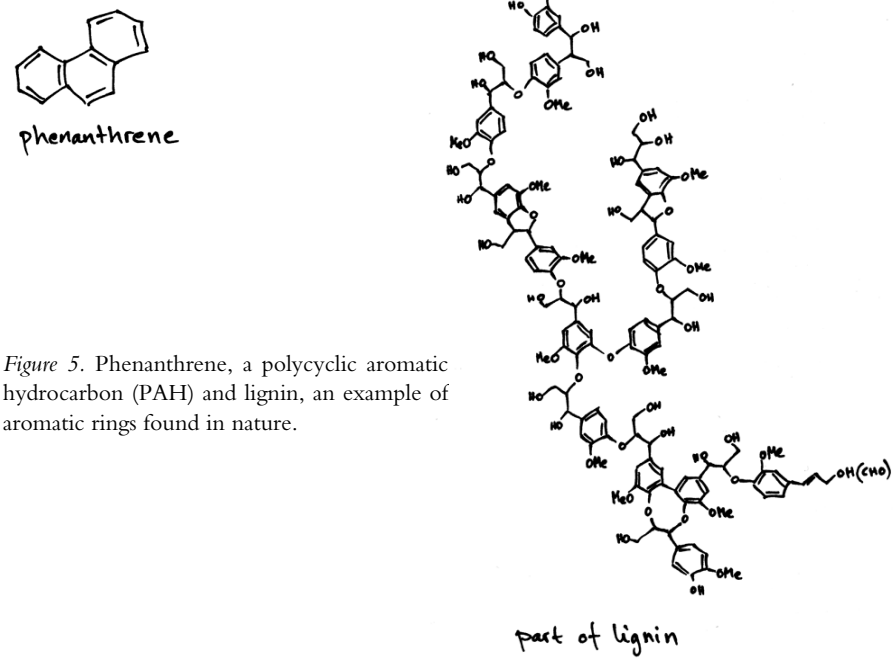

Figure 5. Phenanthrene, a polycyclic aromatic hydrocarbon (PAH) and lignin, an example of aromatic rings found in nature.

Phenols

Phenols, and chlorophenols in general, have been used in refineries, chemical production as well as in pesticides, disinfectants and paint- and wood preservatives (Swoboda-Colberg, 1995). One such wood preservative is creosote, containing PAHs and phenols such as 4-CP. Creosote is still allowed in Sweden for large scale preservation of wood in contact with water as well as for telephone poles. 4-CP is also formed from chlorine bleaching of pulp (from which paper is made), and from chlorination of wastewater and breakdown of phenoxy herbicides such as 2,4-dichlorophenoxyacetic acid (Pritchard *et al.*, 1987). It is also formed when more highly chlorinated phenols are partially degraded (Woods *et al.*, 1989, Madsen & Aamand, 1992). Such highly chlorinated phenols have been used en masse for e.g. preservation of lumber (Swoboda-Colberg, 1995). The compound 4-NP, on the other hand, is a nitroaromatic compound, and is a byproduct in production of some explosives (Spain, 1995, Swoboda-Colberg, 1995). In addition, this compound can also be formed by hydrolysis of e.g. parathion and methylparathion, compounds used as insecticides (Munnecke, 1976, Nelson, 1982).

Phenols can be toxic to cells because they have potential to uncouple cellular respiration (Terada, 1990, Escher *et al.*, 1996). They are lipophilic and therefore normally able to pass the cell membrane. Since they are weak acids a phenolate ion is formed when the hydrogen atom of the hydroxyl group dissociates from the compound. Thus, a phenolate ion can take up a proton outside the cell, and deposit it on the inside, and in this way uncouple cellular respiration. This effect is what makes phenols lethal to some microorganisms when present at concentrations over a certain threshold. This threshold varies with the phenolic compound and species of microorganism. *A. chlorophenolicus*, for example, is tolerant to unusually high concentrations of 4-CP. The strain was isolated from a soil slurry enriched with increasing concentrations of 4-CP, and subsequently found able to degrade up to 350 μg/ml of 4-CP in pure culture (Westerberg *et al.*, 2000) (Fig. 2). No other microorganism has yet been reported to grow on similarly high concentrations of 4-CP.

One kind of phenol, toxic when present as a sole compound, can be even more toxic if present in a mixture with other phenols. This increase in toxicity is sometimes due to the formation of a dimer between a phenol and a phenolate ion, which is most likely to occur if the two phenols have very

different pK_a-values (Escher *et al.*, 2001). *A. chlorophenolicus* is not limited to surviving and growing in the presence of only one compound at a time, but studies of this organism in the presence of three compounds in mixture, 4-NP, 4-CP and phenol, show that it can degrade all three (**Paper II**). However, the common use of phenol as an enhancer of 4-CP degradation is contradicted in *A. chlorophenolicus*, as phenol is the compound degraded last and slowest. There are also indications that the substituted phenols are degraded through a common catabolic route, whereas phenol is degraded through another pathway. In addition, there are indications of possible transport competition between 4-CP and phenol into the cell (**Paper II**). An observation supporting this theory is based on studies of the mutant T99, harbouring a non-functional hydroxyquinol dioxygenase gene rendering it unable to grow on substituted phenols. However, it grows even better than the wild type strain on phenol, which could be due to downstream effects; for example on an adjacent gene required for substrate transport. Results reported in **Paper IV** confirm that this adjacent gene is non-functional in T99. However, several other genes seem to be knocked-out in the mutant too, suggesting that the down-stream effects may have affected regulatory regions as well. Still, the results from the proteomic study (**Paper IV**) confirm the dissimilarities during growth on 4-CP and 4-NP vs. phenol, strengthening the hypothesis of a different catabolic route for the latter compound.

During degradation of halogenated aromatics the halogen ion(s) is often retained until after ring cleavage, which can be a disadvantage because of the increased water solubility of straight-chained compounds compared to ring-shaped ones. The toxicity is often increased, or created, by the presence of one or several halogen ions, and when the toxic compound is cleaved into an open chain it might be more readily transported to e.g. groundwater. In the degradation of 4-CP, and most likely other substituted phenols, by *A. chlorophenolicus*, the substituent ion is removed prior to ring cleavage, which might thus reduce toxicity.

Parathion

The genome sequence of *A. chlorophenolicus* contains two hydrolase genes that indicate parathion degradation abilities (Unell & Jansson, unpublished). Preliminary experiments show that *A. chlorophenolicus* also has the capacity to degrade this compound.

Parathion has been one of the most intensively used organophosphorous insecticides in the world, but due to its high toxicity to mammals it has been banned in large parts of the world. However, it is still in use in developing countries. Under favorable conditions parathion can be microbiologically degraded, but it binds tightly to soil particles and residues may persist in soil for many years (Katan *et al.*, 1976, Daughton & Hsieh, 1977).

The degradation of parathion proceeds through a hydrolyzing step, catalyzed by a hydrolase such as the ones found in the *A. chlorophenolicus* genome. In this step parathion is degraded to 4-NP and diethylthiophosphoric acid (DETP) (Fig. 6).

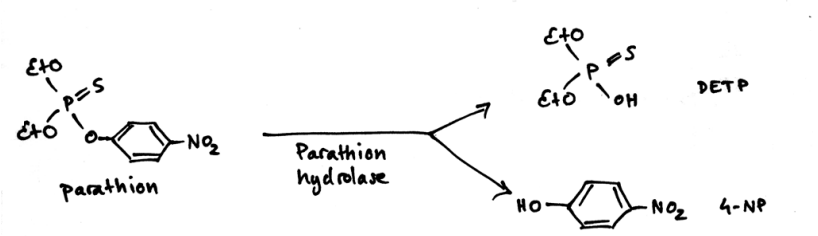

Figure 6. Hydrolysation of parathion into diethylthiophosphoric acid (DETP) and 4-NP.

The degradation of parathion has been reported for co-cultures of at least two different microbial species, where only one can degrade 4-NP further (Karpouzas & Singh, 2006). However, a study of another *Arthrobacter* species suggests that this species can hydrolyze parathion on its own, and utilize the 4-NP formed as a sole carbon source (Nelson, 1982). Thus, it remains to be elucidated how, and to what extent, parathion is degraded by *A. chlorophenolicus*.

Bacterial Adaptation Mechanisms to Stress

As life in the microbial world is characterized by fierce competition and struggle for survival, adaptive stress responses of bacteria are many and diverse, and found in all species. These adaptations require sensitive monitoring of numerous environmental parameters to orchestrate the activities of intricate systems that initiate or readjust cellular responses – and at the same time the delicate balance between cost and gain has to be considered.

A. chlorophenolicus is very stress tolerant, enduring both high concentrations of phenolic compounds and low temperatures, and successfully remediates contaminated soil in laboratory studies (Elväng *et al.*, 2001, Jernberg & Jansson, 2002, Backman & Jansson, 2004). The mechanisms behind the high stress tolerance within this species and its genus, often enabling them to live and prosper in environments too harsh for other genera, is not well known. In the following chapter a few of the mechanisms used by *A. chlorophenolicus* to overcome high concentrations of phenolic compounds, low temperature and general stress associated to life in soil are discussed.

Cell Membrane Adaptations

The cell's first barrier against the surrounding environment is the cell envelope, and thus this is its first line of defense against potential threats from the environment. The Gram positive cell envelope consists of two functional layers: a cytoplasmic membrane surrounded by a thick cell wall of peptidoglycan. Negatively charged teichoic acids (acidic polysaccharides) are attached to the cell wall (Fig. 7). The cell wall maintains the shape of the cell, maintains turgor pressure and is the barrier between the cytoplasm and the immediate environment of the cell (Jordan *et al.*, 2008). The cell wall also has proteins and other molecules that sense and respond to changing conditions in the environment, including nutrient uptake and stress responses.

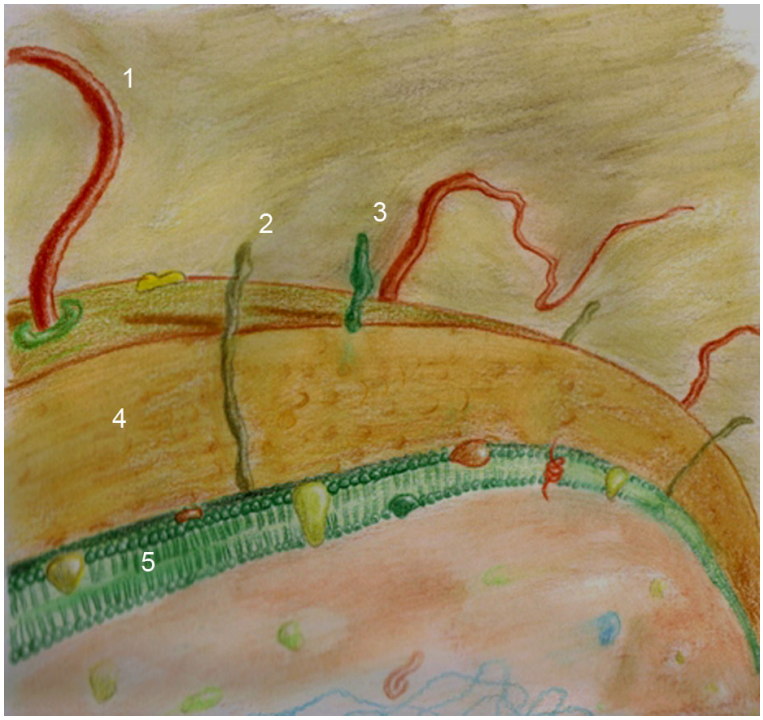

Figure 7. The Gram positive cell envelope. 1) Flagella. 2) Lipoteichoic acid. 3)Teichoic acid. 4) Peptidoglycan layer. 5) Cytoplasmic membrane.

One type of stress commonly encountered by soil bacteria is stress due to nutrient limitation. Many bacteria respond to starvation by reduction in cell size and a change to spherical shapes (Givskov *et al.*, 1994). For example, many *Arthrobacter* species convert from rod-shaped to coccoidal cell shapes. This results in an increase in the relative surface area of the cell, enabling a higher nutrient uptake. In addition, in a starvation study of *A. crystallopoietes* it was found that the coccoid cells contained much more carbohydrate reserves for life-sustaining endogenous metabolism than the rod-shaped cells. The rod-shaped cells were forced to use RNA and protein for their endogenous metabolism to a higher extent than the coccoid cells during a 30-day starvation period (Boylen & Ensign, 1970).

Another type of stress response is that resulting upon exposure to toxic compounds. Some bacteria increase their cell volume when exposed to toxic compounds, in order to decrease the surface to volume ratio (Jordan *et al.*, 2008). By contrast, cells of *A. chlorophenolicus* are smaller and more coccoidal when growing on high concentrations of toxic phenolic substrates compared to when growing on other carbon sources contained in rich media, such as Luria Broth (LB). The reason for this discrepancy remains to be elucidated, but one possibility is that coccoidal cells might be less susceptible to toxins due to changes in the cell envelope. For example, *Arthrobacter* species often show changes in cell membrane fatty acid composition when converting from a rod to coccus shape (Caudales *et al.*, 1998).

Environmental factors such as growth temperature and presence of toxic organic substances have a strong impact on cell membrane fatty acid composition. This phenomenon is well studied in Gram negative bacteria, but more scarcely reported from Gram positives (Sardessai & Bhosle, 2002). Changes in fatty acids are thought to occur in order to maintain a functional bacterial cell membrane regardless of environmental stress. For example the fluidity of the membrane needs to stay in a liquid-crystalline state to keep the cell membrane from cracking or melting; i.e. homeoviscous adaptation (Sinensky, 1974). An additional theory for a change of cell membrane fatty acid composition in response to e.g. changes in temperature is the homeoproton permeability adaptation theory (van de Vossenberg *et al.*, 1999). This theory is based on the important task of the cell membrane as a barrier to protons, resulting in a high proton motive force to enable essential energy transducing processes. According to this theory the viscosity of membranes may change depending on growth temperature (however always

staying liquid-crystalline), but the proton motive force remains constant (van de Vossenberg *et al.*, 1999).

To maintain the homeostasis of membrane fluidity and/or proton permeability, Gram negative bacteria increase the ratio of *trans* to *cis* unsaturated fatty acids in response to an increased temperature or presence of toxic organic compounds (Heipieper *et al.*, 2003, Loffhagen *et al.*, 2004). The few studies performed on Gram positives reveal another approach than the *trans* to *cis* formation in Gram negatives. In Gram positive bacteria it is mainly the ratio of branched fatty acids that is altered; i.e. the *anteiso*:*iso* ratio of branched fatty acids decreases when temperature increases (Fig. 8) (Klein *et al.*, 1999, van de Vossenberg *et al.*, 1999, **Paper III**).

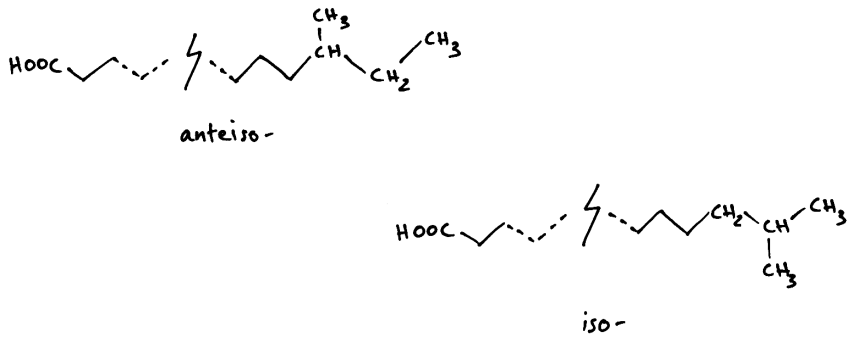

Figure 8. Anteiso vs. *iso*-branched fatty acids.

The effect of temperature and presence of phenolic compounds is described in **Paper III**, where fatty acids from the cell membrane of *A. chlorophenolicus* grown on different phenols and at two different temperatures were analysed.

Stress Related Proteins

When the surrounding environment of a bacterial cell is altered, the protein expression quickly changes accordingly. There are proteins associated with general stress response (universal stress-related proteins, USP's), activated irrespective of the nature of the stress, and there are, for example, specific cold- or heat-shock proteins. The functions of stress proteins are still not fully known, and some results suggest that e.g. cold-shock proteins (CSP's) are important for growth also under optimal temperature conditions (Phadtare *et al.*, 1999), a hypothesis that is strengthened by some of the results presented in **Paper IV**.

Cold shock causes stabilization of the secondary structures in RNA and DNA, resulting in reduced efficiency of translation, transcription and DNA replication. Such deleterious effects are overcome by e.g. cold-shock proteins (Phadtare *et al.*, 1999). A major cold-shock protein, CspA, was first found in *E.coli*, and its homologues have been detected in a variety of Gram negative and Gram positive bacteria, ranging from psychrotrophic to thermophilic strains (Graumann & Marahiel, 1998). A *cspA*-like gene, *capA*, has been reported from studies of the psychrotroph *Arthrobacter globiformis* S155. This gene was expressed rapidly following cold shock, and was still expressed during prolonged growth at low temperature. This makes it a probable cold acclimation protein (CAP) (Berger *et al.*, 1997). Six homologues to *CapA* can be found in the *A. chlorophenolicus* genome sequence, one of which is nearly identical in amino acid sequence to *CapA* of *A. globiformis* (Unell & Jansson, unpublished). However, only one of these genes is consistently over-expressed during growth at 5°C compared to growth at 28°C, when degrading the different substrates 4-CP, 4-NP and phenol whereas the other homologues show a more arbitrary pattern (**Paper IV**). Thus, it seems the putative cold acclimation proteins do have additional roles apart from adjusting to cold growth. For example they might help the cells to adapt to presence of lipophilic hydrocarbons or other toxic compounds (as suggested in **Paper IV**), as well as to drought or starvation.

Apart from the more well-known CSP's and CAP's there is an array of other mechanisms used by microorganisms to survive stressful conditions. In **Paper IV** some proteins are discussed that may be expressed to relieve stress due to temperature changes or exposure to phenolic compounds; such as ATPases with chaperone functions, a protein involved in glycerol metabolism, an uncharacterized conserved protein YceI, and others.

Dormancy

The ability of bacteria to survive in hostile environments is essential to their persistence. Some survive harsh conditions for long periods of time by forming resistant spores, but some non-sporulating bacteria can also persist by forming cells with very low metabolic activity. This state is called dormancy. The term "Viable But Non Culturable" (VBNC) is also sometimes used for cells in this state, and there is currently much confusion and contradiction regarding the distinction between the two terms. In this thesis the term "dormancy" is used throughout for this reversible state.

Sometimes previously unculturable dormant cells can be coaxed back into culture, a phenomenon known as resuscitation. In the actinobacterium *Micrococcus luteus* a protein was found that was secreted by dividing cells and that increased the number of colonies that could be formed by previously dormant cultures (Mukamolova *et al.*, 2002). This protein was called *r*esuscitation *p*romotion *f*actor (Rpf). Homologues for this gene have been found in several species, and one homologue is also found in the *A. chlorophenolicus* genome (Unell & Jansson, unpublished) as well as in the other two *Arthrobacter* species sequenced to date. The Rpf protein seems to have a general role as a bacterial growth factor or a cytokine (Mukamolova *et al.*, 2002). The conserved domain of Rpf shows homology to lysozyme and to the glycosylase domains of certain transglycosylases (Cohen-Gonsaud *et al.*, 2005), both known to cleave peptidoglycan. Thus, it seems the Rpf protein catalyzes cell wall peptidoglycan cleavage, and thereby resuscitates bacteria. The mechanism behind this process is not yet known.

In previous studies the physiological status of *A. chlorophenolicus* was investigated, revealing that the cells became largely dormant when incubated at 5°C, but during incubation at 28°C most of the cells died (Backman *et al.*, 2004). Upon addition of nutrients, some of the dormant cells incubated at 5°C were rapidly resuscitated, but for the cells incubated at 28°C no change in the number of active cells was observed (Backman, 2004, Backman *et al.*, 2004, Maraha, 2007). These results indicate that *A. chlorophenolicus* survives better in soil incubated at low temperatures, which has been seen also in other bacteria (Duncan *et al.*, 1994, Curras *et al.*, 2002), a phenomenon that could be explained by the theory that proteins induced by stress, e.g. cold, may cross-protect the cells against nutrient deprivation or other kinds of stresses found in the soil environment (Givskov *et al.*, 1994). The hypothesis of cross-protection is supported in **Paper IV**, where several proteins changing in amount depending on growth temperature also changed due to the toxicity of the phenolic compound present.

Arthrobacter Genomics

To date, three genomes of *Arthrobacter* species have been sequenced: *A.aurescens* TC1 (Mongodin *et al.*, 2006), *Arthrobacter* sp. FB24 (http://genome.jgi-psf.org/finished_microbes/art_f/art_f.home.html, http://genome.ornl.gov/microbial/arth/) and *A. chlorophenolicus*, of which the latter is still a draft sequence (http://genome.ornl.gov/microbial/achl/, Unell & Jansson, unpublished).

The genome of *A. chlorophenolicus* is 4.66 Mb in size and consists of one circular chromosome and two large plasmids, together containing 5286 genes. The G+C content is 66%, which is slightly higher than the other two *Arthrobacter* species sequenced to date (Table 1). Although there are many similarities between the 3 species, there are also many differences. For example, 75% of the 5375 genes in the *A. chlorophenolicus* genome have a top BLAST match to one of the two other closed *Arthrobacter* genomes – *A.aurescens* TC1 and *Arthrobacter* sp. FB24. In the *A. chlorophenolicus* genome as many as 773 genes (14.4%) could have a potential horizontal gene transfer (HGT) origin since they show top BLAST hits to other genera than *Arthrobacter*. 491 genes (9.1%) have no hits to other genomes at all, which suggests a specialized niche for this bacterium. The corresponding number for the *A.aurescens* TC1 genome is 13.2%, underlining the high diversity in metabolism and ecology within the genus. A summary of the comparisons between the three *Arthrobacter* genomes is found in Table 1. Interestingly, the *A. chlorophenolicus* genome is the smallest but still contains more genes than the other species.

Table 1. *Comparison of general features between genomic sequences of* A. chlorophenolicus, A.aurescens *TC1 and* Arthrobacter *sp. FB24.*

Genome name	Bases (Mb)	Genes	GC (%)	Pseudo genes	Cell motility (%)	Defense (%)	Intra-cellular trafficking (%)
A.aurescens TC1	5.23	4660	62	0	0.08	1.36	1.09
A. chlorophenolicus	4.66	5375	66	2	1.17	1.64	1.91
Arthrobacter sp. FB24	5.1	4605	65	30	0.08	1.28	1.28

Concerning the genes for xenobiotic degradation and metabolism present in KEGG categories, the *A. chlorophenolicus* genome contains fewer such genes than the other two species; 40 genes (6% of the genome) compared with 54 and 85 genes (7% and 8% of the genomes) for *A. aurescens* TC1 and sp. FB24 respectively. According to KEGG categories there are no indications of xenobiotic degradation abilities unique for *A. chlorophenolicus* compared with the other two species, but the presence of single genes in *A. chlorophenolicus* indicates possible degradation of e.g. parathion and cocaine that are not found in the other two species. Degradation of the former has been confirmed in preliminary experiments (Unell & Jansson, unpublished).

Previously, a *cph* (*c*hloro*ph*enol degradation) gene cluster was sequenced and characterised in *A. chlorophenolicus* (**Paper I**). Its significance for 4-CP degradation was, among other methods, confirmed through the construction of a mutant (T99) with a non-functional 1,2-hydroxyquinol dioxygenase gene (*cphA-I*). This mutant is unable to grow on substituted phenol and produces a red pigment when incubated with these compounds. However, the mutant strain grows well on phenol. This indicates a different or additional pathway for the latter compound compared to the substituted phenols (**Paper II**). Proteomic analyses reported in **Paper IV** show many differences between protein expression between the mutant and the wild type, and between growth on substituted phenols vs. phenol. These results strengthen the hypothesis of a different pathway for phenol than for substituted phenols.

The 4-chlorophenol degradation gene cluster found in *A. chlorophenolicus* is not conserved in gene order in the other two species, but most of the genes present in the cluster have homologues in the other species' genomes. However, a transcriptional activator domain present in the *A. chlorophenolicus* *cph* gene cluster is absent in the other *Arthrobacter* genomes, and in the *A.*

aurescens TC1 genome an ABC molybdate transporter (*cphX* in *A. chlorophenolicus*) is missing. The *cph* homologues are found dispersed in the *A. aurescens* TC1 genome, but in the *Arthrobacter* sp. FB24 genome several are collected in a cluster. This cluster show similarities to the *cph* gene cluster in *A. chlorophenolicus*, and three of the genes in the cluster, *cphC-II, cphF-I* and *cphF-II*, are highly conserved between the species. However, the duplicated *cphF* genes in *A. chlorophenolicus* only have one homologue in *Arthrobacter* sp. FB24. A comparison between the clusters is seen in Fig. 9.

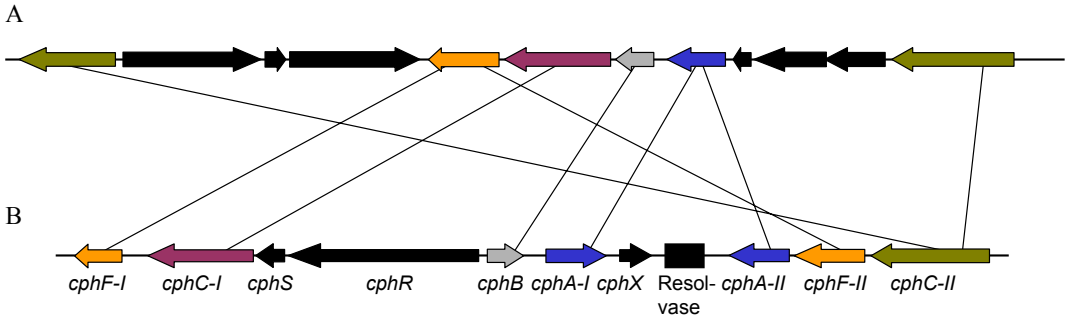

Figure 9. Comparison of part of the *Arthrobacter* sp. FB24 genome (A) with the *cph* gene cluster in *A. chlorophenolicus* (B). *cphF-I*, *cphF-II* and *cphC-II* are highly conserved between the species.

In **Paper I** we discuss indications of a horizontal gene transfer event in the *cph* gene cluster of *A. chlorophenolicus*, a probable cause to the duplication of genes *cphA*, *-C* and *-F*. For example, the G+C content is significantly lower in the genes to the left of the resolvase pseudogene seen in Fig. 9 compared to the genes to the right, and to the rest of the genome. The *cph* homologues found in the other two *Arthrobacter* species all have the high G+C content characteristic for this genus. When comparing the number of homologues to the *cphA*, *-C* and *-F* genes, *A. chlorophenolicus* has a higher copy number of these genes in the draft genome sequence. Some of these genes were already identified in the *cph* gene cluster in **Paper I**, but not all. *A. chlorophenolicus* has 3 copies of the maleyl acetate dehydrogenase *cphF* genes, while *Arthrobacter* sp. FB24 and *A. aurescens* TC1 have 1 copy. The *cphC* monooxygenase genes count to 6 copies in the *A. chlorophenolicus* genome, whereas *Arthrobacter* sp. FB24 has 5 and *A. aurescens* TC1 has 4. The *cphA*

genes, encoding ring-cleaving dioxygenases, are 3 in the *A. chlorophenolicus* genome, 2 in *Arthrobacter* sp. FB24 and 1 in *A. aurescens* TC1.

All three *Arthrobacter* species contain genes for both synthesis and catabolism of trehalose and glycogen, as well as glycogen synthetic branching enzymes. This is one explanation for the remarkably high stress-tolerance within the *Arthrobacter* genus, because these compounds are known osmolytes and reserve energy sources, respectively. A previous study of *Arthrobacter globiformis* stressed the high concentrations of glycogen and trehalose in the cells as one of the major reasons for the genus' resistance to nutrient starvation, desiccation and osmotic stress (Zevenhuizen, 1992).

Another survival strategy found in e.g. *A. aurescens* TC1, shared by *A. chlorophenolicus*, is the presence of an unusually large number of oxidase genes (30, 37 and 34 genes in *A. chlorophenolicus*, *A.aurescens* TC1 and *Arthrobacter* sp. FB24, respectively) (Mongodin *et al.*, 2006, Unell & Jansson, unpublished). These genes enable the cell to survive the reactive oxygen radicals generated by the intense aerobic metabolism. There are also several cupin associated genes: 6, 9 vs. 9 genes in *A. chlorophenolicus*, *A. aurescens* TC1 and *Arthrobacter* sp. FB24, respectively. Cupins, a superfamily of β-barrel structural domains, are thought to be involved in stress responses, desiccation tolerance, cell wall structure etc. (Dunwell *et al.*, 2000). The cupin superfamily includes several dioxygenases and plant-associated germins (Dunwell *et al.*, 2000). In addition, within all three *Arthrobacter* genomes several putative cold shock proteins can be found (6, 5 and 6 genes in *A. chlorophenolicus*, *A. aurescens* TC1 and *Arthrobacter* sp. FB24, respectively).

In similarity with the other two *Arthrobacter* strains, *A. chlorophenolicus* contains many regulatory genes (195 genes), and the most common class of transcriptional regulators are TetR (36 genes), GntR (20 genes) and LysR (19 genes). TetR transcriptional regulators control genes for multidrug resistance, enzymes implicated in different catabolic pathways, biosynthesis of antibiotics, osmotic stress and pathogenicity of bacteria (Ramos *et al.*, 2005). The GntR regulators have been found to act as dimeric repressors and/or activators that respond to effector molecules, often carboxylate-containing intermediates in primary metabolism (Hoskisson *et al.*, 2006). LysR transcriptional regulators are the largest family of prokaryotic regulators, and activate the transcription of operons and regulons involved in extremely diverse cellular functions including nitrogen fixation, oxidative stress response and bacterial virulence (Schell, 1993). A gene typical of a LuxR regulator is found within the cph gene cluster (formerly given gene designations *cphS* and *cphR*; Nordin et al., 2005). The LuxR family is typical

of quorum sensing systems and it would be of interest to determine if this process occurs in *A. chlorophenolicus*.

There are substantially fewer transposases in the *A. chlorophenolicus* genome (3 genes) than in *A. aurescens* TC1 and *Arthrobacter* sp. FB24 (35 vs. 13 genes).

243 genes with known function (4.5% of the genome) are unique for *A. chlorophenolicus* compared with the other two *Arthrobacter* species. A striking difference between the three *Arthrobacter* species is the presence of flagella in *A. chlorophenolicus*, which are missing in the other two species. The *Arthrobacter* genus consists of both motile and non-motile species, however the majority of described species are non-motile (Holt, 1994). When studied under the microscope the motility of *A. chlorophenolicus* seems to be induced during the rod phase of growth, while coccoid cells are non-motile. This is consistent with findings of Stanlake and Clark (1976) concerning two other *Arthrobacter* species. In an electron microscope it is evident that coccoid cells of *A. chlorophenolicus* are not flagellated, while the rod-shaped cells show remnants of peritrichous flagella or pili (Unell & Jansson, unpublished; Fig.10). Possibly the flagella were broken during the preparation and stabilisation of the cells prior to microscopy.

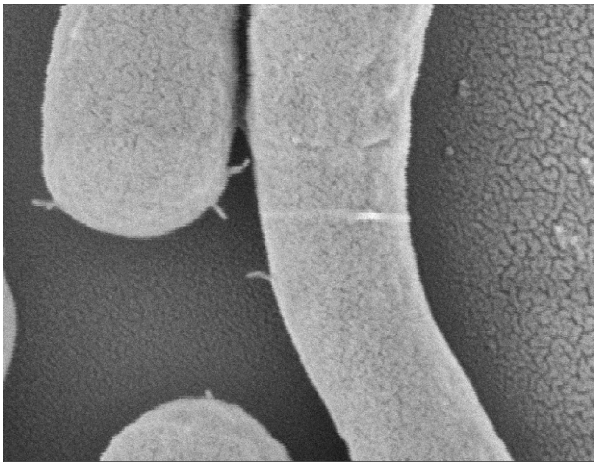

Figure 10. Picture of remnants of flagella or pili in log phase *A. chlorophenolicus* cells.

Other major groups among the genes with known function, unique for *A. chlorophenolicus* compared to the other *Arthrobacter* species, are 29 genes coding for carbohydrate transport and metabolism, 22 genes for DNA replication, recombination and repair, and 12 for defense mechanisms (Table 1). Several genes for xylose and pectate degradation as well as two genes indicating possible parathion degradation are interesting findings in this list, and preliminary studies confirm xylose and parathion degradation (Unell & Jansson, unpublished). Several of the unique genes consist of members of the Type II secretion system, a pathway for secretion of virulence determinants or degradative enzymes such as pectinases, cellulases and proteases. Unique for the *A. chlorophenolicus* genome, compared to the other *Arthrobacter* species, are also several genes indicating resistance against heavy metals such as mercury and arsenic, as well as a plasmid maintenance killer system and the antidote protein associated with this plasmid maintenance system. A large part of the defense genes unique to *A. chlorophenolicus* compared to the other two *Arthrobacter* species are antibiotic resistance genes or antibiotic biosynthesis genes, and there is also a gene encoding an insecticidal toxin (Unell & Jansson, unpublished). In addition, among the genes found in *A. chlorophenolicus* but not in the other two species are some genes associated with plant interactions. There are publications of plant endophytic *Arthrobacter* species (Moore *et al.*, 2006, Sziderics *et al.*, 2007), and the significance of these genes in *A. chlorophenolicus* remains to be studied.

As a conclusion, the genome of *A. chlorophenolicus* answers many questions posed in the past, but even more questions have arisen – the paradox of science.

Methods

In the world of molecular biology, technique development is rapid in some areas, while for some experiments the traditional way of studying a phenomenon is still the best, or even the only one existing. For the papers in this thesis I have been using several relatively traditional molecular methods to study the genetics and degradation behaviour of *A. chlorophenolicus*, and I will not describe these in detail here. Instead, in this chapter I will focus on some of the more novel approaches used in **Paper IV**. For example, shotgun proteomics is a new powerful, high-throughput approach for obtaining information about proteins expressed in bacteria. This methodology has recently been greatly improved due to advances in mass spectrometry (MS) equipment and parallel advances in computational software. However, to be able to use this method, the full genome sequence of the organism studied must be available.

Genome Sequencing of *A. chlorophenolicus*

The funding by the DOE Joint Genome Institute made the dream of the complete *A. chlorophenolicus* genome come true. The task was completed through the use of Sanger sequencing and the 454 Life Science Genome Sequencer™ system (Roche, Basel, Switzerland). Frederick Sanger developed Sanger sequencing, a chain terminating sequencing method using modified nucleotides to reveal the nucleotide sequence (Sanger *et al.*, 1977). This approach relies on a low concentration of a chain terminating nucleotide (most commonly a di-deoxynucleotide) in the mixture of template, primer and deoxynucleotide bases. This nucleotide occasionally terminates elongation by the DNA polymerase, which results in DNA

fragments of different lengths, all having the terminating nucleotide in the end. Using the four different bases as terminating nucleotides makes it possible to deduce the sequence through size separation of the different DNA fragments.

The 454 sequencing method is a form of pyrosequencing (Ronaghi *et al.*, 1998) where millions of copies of DNA can be sequenced in parallel (Margulies *et al.*, 2005). Pyrosequencing was developed in Stockholm in the 1990's, using a chemiluminescent reaction. In this approach, the four different bases are added sequentially, and as the DNA polymerase synthesises the complementary strand, one base pair at a time, light is emitted only when the nucleotide is incorporated into the strand. This allows for sequence determination of the DNA. In 454 sequencing, a high-throughput pyrosequencing method, single stranded DNA libraries are immobilised onto small beads in a water-in-oil emulsion. This results in microreactors each containing one bead with a unique DNA library fragment. Each library fragment is amplified through emulsion PCR. Subsequently the emulsion is loaded onto a PicoTiterPlate, allowing only one bead per well. The following pyrosequencing reaction determines the sequence for each of the hundreds of thousands of beads with millions of copies of DNA, in parallel (Margulies *et al.*, 2005).

The full genome sequence of an organism opens enormous opportunities for exploration of its physiology, catabolic abilities and array of adaptation mechanisms. However, the genome does not change following changes in e.g. environmental conditions, thus the responses and actions taken to different external stimuli cannot be revealed by studying the genome alone. One possibility is to study the transcriptome, or genes that are transcribed into RNA under different conditions. Ultimately, the products of expression are proteins and the resulting proteome will be different depending on the particular growth requirements and needs of the cell. Expressed in another way, the genome is static while the transcriptome and proteome are dynamic. However, knowledge about the genome is necessary for the study of the complete proteomes, by use of for example "shotgun proteomics". In **Paper IV** of this thesis a shotgun proteomics approach was used to study proteome alterations in *A. chlorophenolicus* in response to different growth temperatures and to different phenolic compounds.

Proteomics

The science of proteins – proteomics – has recently become more feasible after the advent of full genomic sequences and thereby databases containing the full list of translated proteins from these genomes. Until recently, proteomics was highly labour-intensive and relied on the use of 2-dimensional polyacrylamide gel electrophoresis (2D-PAGE). In this method the proteins are first separated by their isoelectric points (1st dimension), and in a following step by their molecular masses (2nd dimension). If the separation is good, each spot consists of one protein that can be excised and subsequently sequenced through Edman degradation or, more recently, through *de novo* sequencing using mass spectrometry (MS). A 2D-PAGE gel can visualize 1000-1500 protein spots for further sequencing, but the subsequent sequencing of the proteins is time consuming. In addition, specific classes of proteins are known to be under-represented or excluded in 2D gels, and one spot often contains several proteins (Peng & Gygi, 2001). Still, the 2D-PAGE gel technique is the dominant method in use for bacterial proteomics.

Shotgun proteomics

The full genome sequence of *A. chlorophenolicus* opens the possibility of using a different proteomics approach; namely shotgun proteomics, also called bottom-up proteomics. In this approach, the total proteome expressed under a specific condition can be assessed; i.e. thousands of proteins in a short time. Shotgun proteomics relies on highly precise and accurate MS measurements of intact and fragmented peptides. MS has historically been important for mass measurement and structural information of small molecules, but technical advances in recent decades have made MS applicable to large biomolecules such as proteins, nucleic acids and their complexes.

All mass spectrometers share three fundamental components: 1) the ion source, where gas-phase ions are generated from the sample, 2) the ion analyzer, where the charged particles are separated or sorted by their mass/charge ratios and 3) the ion detector, where the abundances and mass/charge values are measured (VerBerkmoes, 2004).

To be able to perform MS analyses, gas-phase ions must be formed, and the ability to form such ions from larger molecules has been achieved in the electrospray ionization technique (ESI) (Fenn *et al.*, 1989) or matrix-assisted laser desorption/ionization (MALDI) (Hillenkamp *et al.*, 1991, Nakanishi *et al.*, 1994). For the proteomic analyses performed on *A. chlorophenolicus*, ESI was used. ESI utilizes a high voltage needle to transfer preformed ions from solution to gas phase (VerBerkmoes, 2004).

Mass spectrometers today have the resolving power to simultaneously differentiate very complex mixtures. However, peptides are not equally easily ionized, thus the more easily ionized species will be overrepresented in abundance in the mass spectra. This competition for charge in the ionization process results in suppression of the more poorly ionized species to the extent that they might not be detectable. This is termed *ion suppression*. One solution to this dilemma is to couple a separation stage, e.g. chromatography, with the mass spectrometer. The common use of high performance liquid chromatography (HPLC) coupled to ESI-MS provides partial purification of the peptides, so that they enter the mass spectrometer at different times. This partially avoids the ion suppression problem, as fewer peptides are measured at the same time.

Even though accurate molecular mass and limited fragmentation are achievable for intact proteins, the identification of the complete amino acid sequence of the protein is often difficult to make based on this information. By using proteolytic digestion of the protein into peptides more extensive sequence information is provided. The most common protease employed is trypsin, cleaving proteins at arginine and lysine residues.

The *A. chlorophenolicus* proteins' paths towards designation by the help of shotgun proteomics can be described as follows:

- Trypsin degradation, adding positive charges to both ends of the resulting peptides.
- Separation of peptides by charge (ion exchange chromatography).
- Separation of peptides by hydrophobicity (reverse-phase chromatography).
- Transformation to gas-phase ions through needle-tip with high voltage (3.6 kV) applied (ESI).
- Ions enter the ion trap, where the instrument performs a full scan MS of the ions' mass/charge ratio (m/z). The 5 most abundant peaks from the full scan are fragmented (MS/MS or tandem mass spectrometry).
- Data processing and bioinformatics.

The mass spectrometer performs both full scan MS and subsequently MS/MS (fragmenting) of the most abundant m/z peaks; i.e. the instrument oscillates between the two functions. A process called dynamic exclusion makes sure an ion with the same mass/charge is not fragmented twice. To do that, the mass/charge values of all fragmented ions are put on a list, which can contain up to 300 posts. The ions on this list are not fragmented again.

After the completion of the mass spectrometry, all peptides' mass/charge ratios and fragmentation patterns are compared to the translated genome sequence of the species, which is cleaved and fragmented *in silico*. One of the most widely used and accepted search algorithms for this task is SEQUEST (Eng *et al.*, 1994), but there are several other programs performing the same procedure, e.g. DBDigger (Narasimhan *et al.*, 2005, Tabb *et al.*, 2005). The latter has the advantage of being very fast and identifying more peptides and proteins than SEQUEST, but has a higher rate of false positives (1-5% compared to 1% for SEQUEST). In the proteomic analyses of *A. chlorophenolicus* DBDigger was used (**Paper IV**). An outline of the shotgun proteomics approach is illustrated in Fig. 11.

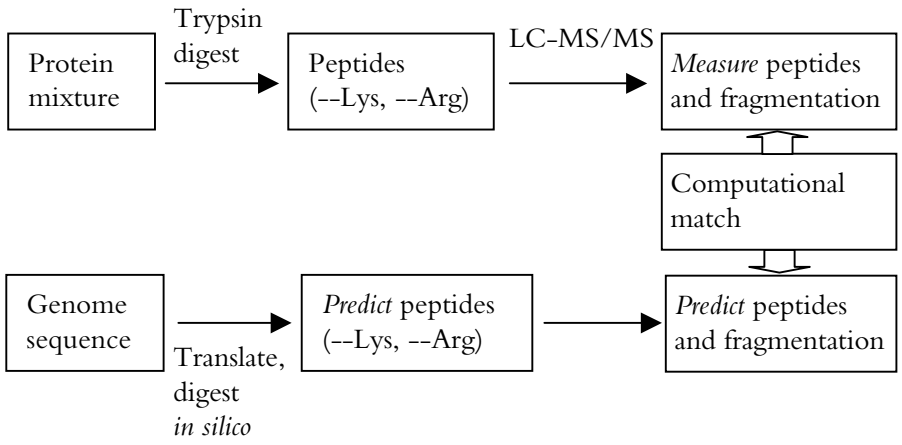

Figure 11. Outline of the shotgun proteomics method. (Courtesy of Jill Banfield, Michael Thelen & Nathan Verberkmoes)

The tens of thousands of MS/MS spectra from a single experiment must be filtered and sorted in order to be able to extract useful information from them. To obtain a list of proteins that do not have an overrepresentation of e.g. false positives, the algorithm DTAselect (Tabb *et al.*, 2002) can be used. Using this program, thresholds for e.g. sequence coverage (percent of a given protein that was covered by sequence information from fragmented ions), filters off any unlikely proteins found. The number of peptides matching a protein is also an issue, since it is likely that a protein matched only by one peptide could be a false positive.

After filtering the data using DTAselect a list of proteins most likely present in the sample is obtained. To compare this list with other samples there are several computer programs or cluster analyses that can be used, for example the program Contrast (Tabb *et al.*, 2002).

Conclusions

During the work of this thesis, some major findings about *A.chlorophenolicus* physiology, genetics and proteomics were revealed as listed below.

- *A. chlorophenolicus* uses an unusual and effective catabolic pathway for degradation of 4-chlorophenol.
- A gene cluster responsible for 4-CP degradation was cloned, sequenced and characterised, and a mutant containing a non-functional ring-cleavage gene in the pathway was constructed.
- The 4-CP degradation pathway is used for degradation of phenol substituted with several different halogen ions in the para-position, but not for degradation of phenol.
- *A. chlorophenolicus* is capable of complete degradation of mixtures of three different phenols. They are degraded in the order 4-NP>4-CP>phenol, i.e. in decreasing order of toxicity. The substituted phenols are degraded through the same degradation pathway whereas phenol is degraded through another or an additional route.
- *A. chlorophenolicus* adapts to changes in temperature and presence of phenolic compounds by changing the membrane fatty acid composition of *anteiso*- and *iso*-branched fatty acids.
- The genome of *A. chlorophenolicus* revealed properties such as parathion and cocaine degradation, defense functions, motility and genes commonly found in association with plants.
- The proteomes of *A. chlorophenolicus* grown under different growth conditions revealed complex adaptation mechanisms both in response to growth temperature and substrates.

In summary, this is an organism adapted to a harsh environment, and still the mechanisms employed to do so are largely unexplored. The versatility of the degradative abilities of *A. chlorophenolicus* in conjunction with the tolerance to starvation, as well as to presence of high concentrations of phenolic compounds, is impressive. Some of these traits, presumably also at least some of the defense mechanisms found in the genomic sequence, are probably shared with other members of the *Arthrobacter* genus. This makes the genus' high abundance in soil very reasonable. However, the selection pressure put on *A. chlorophenolicus* during the selective enrichment preceding its isolation might have caused alterations on the genome level, giving this species new abilities not shared by many others in the genus. The further studies of the genomics, physiology, transcriptomics and proteomics of *A. chlorophenolicus* will be interesting to follow.

Future Perspectives

It has been an adventure to follow the inscrutable bug *A. chlorophenolicus* from the first wearying efforts to sequence the *cph* gene cluster piece by piece, until getting the entire genome at once into my computer and thereby also the opportunity to study its complete proteomes under various conditions. What could these enormous amounts of data, together with all the physiological studies performed on this species, pour into the hands of the future?

There are numerous exciting ways to continue the research on *A. chlorophenolicus*. The genes found in this species, such as additional degradation genes, genes indicating plant association, defense mechanisms etc. all hide their own secrets. In the best of futures all of these areas would be carefully studied. The parathion degradation abilities could be studied both in terms of the concentrations tolerated by *A. chlorophenolicus* and the degradation pathway used. Possible plant association functions need to be studied further in the literature before deciding on suitable plants to grow in association with *A. chlorophenolicus* and to study the resulting effects. The results of the proteomic study leave many open doors to explore, of which only a small part is the degradation pathway(s) of phenol and the gene regulation mechanisms.

A natural continuation of the studies of the mutant T99, that has an inactivated hydroxyquinol dioxygenase gene, (**Paper II** and **IV**) would be to entangle how the mutation effects down-stream genes such as the probable transporter gene adjacent to the mutated gene. In our back pocket there are also 9 other mutants unable to grow on 4-CP that just wait to be studied further. Molecular tools to study actinobacteria have been scarce to date, but every now and then new methods are reported, e.g. a recently described actinobacterial vector (Miteva *et al.*, 2008) opening new doors for more in depth studies of *A. chlorophenolicus* and other actinobacteria.

"This writing business. Pencils and what-not. Over-rated, if you ask me. Silly stuff. Nothing in it."

– Eeyore, "Winnie-the-Pooh", A. A. Milne –

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Nu ska jag ut och känna våren!

I somnar ska jag snarkbada.

I röst ska jag lyssna på löven.

I hår ska jag kamma gräset.

I slintras råkade jag säga snöskorpa

och det fick jag, fast jag vet att det

heter skare.

- Magnus Lönn -